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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Richard X. Lyn-Cook

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Abstract

CORTICAL DEVELOPMENT IN FIBROBLAST GROWTH FACTOR 2 KNOCKOUT MICE. Richard X. Lyn-Cook (Sponsored by Flora M. Vaccarino, M.D., Child Study Center, Yale University School of Medicine, New Haven, Connecticut)

The pathogenesis of a number of disorders that lead to mental retardation and cognitive deficiencies are poorly understood. These disorders are often associated with a decrease in the number of cortical neurons. Fibroblast growth factor-2 has been strongly implicated in the regulation of cortical volume and cell number. This experiment is designed to measure the values for the proportion of proliferative cortical cells (proliferative, P fraction), and those that exit the cell cycle (quiescent, Q fraction) in FGF-2 knock-out mice. Embryonic days E11.5, 13.5, and 15.5 were examined covering nearly the entire period of neurogenesis (E11-E17). The results of this experiment could further elucidate the role of FGF-2 in control of cortical development.

Acknowledgements

This project would not have been possible without the support and guidance of Dr. Flora Vaccarino. Special recognition is also extended to Rossana Raballo and Julianne Rhee for all their help in the laboratory.

I would also like to thank Steven Williams, Rowan Reid and last but not least, my parents, brother and sister.

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Introduction

The human cerebral cortex is the evolutionary pinnacle of the mammalian central nervous system. It comprises about two thirds of the neuronal mass of the central nervous system and a majority of its synapses (Rakic, 1988). As we evolved, the cortical surface area and neuronal number increased with associated increases in intelligence, higher cognitive functioning, and more complex forms of behavior (Bayer and Altman, 1991).

Despite the differences in neuronal number and cortical volume between humans and lower mammals, the general pattern of cerebral cortical development is similar (Rakic, 1988, 1995). For this reason, studies in primates and rodents have proved invaluable in the search for clues to the processes that govern neurogenesis. These studies have also shed light into the pathogenesis of clinical disorders that result from aberrant cortical development.

Neuronal progenitor cells arise from the pseudo-stratified ventricular epithelium (PVE) lining the lateral ventricles in the embryonic telencephalon. Cortical development occurs in stages divided into neurogenesis, neuronal migration and neuronal differentiation (Rakic, 1988; Caviness *et al*, 1995). The complexity of cortical development makes it quite prone to disruptions at one or more of its stages. This can result in a host of abnormalities including mild to severe forms of mental retardation and poor intellectual attainment (Berger-Sweeney *et al*, 1997). Disrupted cortical development may lead to microcephaly. Microcephaly in children has been linked with mental retardation though it is not an absolute prerequisite for this condition (Martin, 1978; Gross, 1978). Animal models of microcephaly involving the injection of the antimitotic agent methylazoxymethanol (MAM) into pregnant rat and mouse dams at the peak of

neurogenesis have yielded a 50% decrease in cortical thickness in the offspring. Furthermore, these animals were found to exhibit motor hyperactivity, and cognitive deficits depending upon the complexity of the task. These animals did perform normally in other behaviors such as startle, righting, mothering and visual discrimination, however (Berger-Sweeney *et al*, 1997)

A 50% decrease in cortical thickness in a rat or mouse may not be devastating to its survival, whereas a similar finding in humans could result in severe functional deficits. Congenital anopthalmia and medial geniculate body malformation, have been linked to a decrease in the size of the visual and auditory cortices, respectively (Rakic, 1988). Humans have evolved to depend on higher cortical functions much more for survival.

The basic molecular mechanisms of cortical development are still poorly understood. In order to gain insight into thepathogenesis and treatment of disorders stemming from aberrant cortical development, we need to elucidate the basic mechanisms of and the factors that influence neurogenesis. The fibroblast growth factor (FGF) family of proteins has been associated with a host of physiological and pathological processes and has been shown to influence development (Bikfalvi *et al*, 1997).

Background and Review of Literature

Basic fibroblast growth factor (FGF2) is a potent mitogenic and neurotrophic agent that induces proliferation of cells of mesodermal and neuroectodermal origin (Powell *et al*, 1991). It is involved with angiogenesis, smooth muscle growth, wound healing, tissue repair, hematopoiesis and has been demonstrated to affect survival, proliferation and differentiation of a

variety of cell types in the central nervous system. (Bikfalvi *et al*, 1997; Cavanagh *et al*, 1997). The biological functions of FGF2 are mediated via binding to high affinity tyrosine kinase receptors (Partanen *et al*, 1993).

During neuronal development, FGF2 has been detected in a variety of species (Bikfalvi *et al*, 1997) In mice, messenger RNA for FGF2 appears by E9 to E9.5 (Nurcombe et al, 1993; Ortega *et al*, 1998). *In vitro* studies have shown that FGF2 delays cortical progenitor cell differentiation without affecting cell cycle parameters (Cavanagh *et al*, 1997). Earlier *in vitro* studies demonstrated that FGF2 stimulated proliferation of cortical neuroectodermal cells (Temple *et al*, 1995; Gensburger *et al*, 1987; Vaccarino *et al*, 1995), maintains the survival of single cultured neurons (Unsicker *et al*, 1992), and induces proliferation of committed neuronal precursor cells (Temple *et al*, 1995) and mature oligodendrocytes (Grinspan *et al*, 1993). Otx2 is one of several homeobox genes expressed in different regions of the developing mammalian forebrain and is critically involved in forebrain development. Otx2 mRNA level was enhanced in cells from rat telencephalon exposed to FGF2 *in vitro*, suggesting that FGF-2 may play a role in forebrain neurogenesis (Robel *et al*, 1995).

In vivo studies have also shown a promising role for FGF-2 in neurogenesis. Neutralizing antibodies to FGF2 reduced the number of cerebellar granule and hippocampal precursor neurons *in vivo* (Tao *et al*, 1997).

There were several studies using FGF2 knockout mice. One suggested that FGF2 has a role in the migration and differentiation of neuronal cells, but no appreciable effect on proliferation in contrast to the *in vitro* studies (Dono *et al*, 1998). The knockout mice used in this study are viable and fertile yet their data suggests that the adult mice have striking defects in cortical morphometry,

which is not a result of decreased proliferation. Another study by Ortega *et al* demonstrated a decrease in neuronal density in the motor cortex of FGF2 -/- versus +/+ control (Ortega *et al*, 1998) in contrast to the Dono results. Data obtained in our laboratory revealed a 61% decrease in total neuron number in adult cerebral cortices of FGF2-/- mice (Vaccarino *et al*, 1999). In addition, embryonic rats injected in the cerebral ventricles with a single dose of FGF2 at the beginning of neurogenesis (E15.5) showed a 53% increase in volume and 70% increase in total number of neurons at E20.5. Similarly microinjected rats assayed at adulthood showed an 87% increase in total number of neurons (Vaccarino *et al*, 1999).

The number of progenitor cells, rate of proliferation, and number of mitotic cycles before terminal differentiation play a significant role in corticogenesis. The rate of proliferation is measured as length of cell cycle, and the proportion of dividing cells as growth fraction. Cortical neurogenesis occurs from embryonic day 11 through early 17 (E11-E17) in the mouse. During this period, there are approximately 11 cell cycles with a growth fraction of 1.0 (Takahashi *et al*, 1995, 1996). But what effect if any does FGF2 have on these parameters? Preliminary data from our laboratory demonstrate that FGF2 knockout mice have a decreased proportion of dividing cells (growth fraction) compared to controls. Conversely, rats microinjected with FGF2 at E15.5 demonstrated an increased growth fraction compared to vehicle microinjected controls. In both cases the length of the cell cycle between treated and controls was unchanged (Vaccarino *et al*, 1999). The reasons for the changes in growth fraction are not clear, nor do we know about the numbers of proliferative cells and/or changes in the number of integer cell cycles in FGF2 knockout mice. It is

possible that the number of proliferating cells is lower to begin with in FGF2 knockout mice, and that fewer proliferating cells require fewer cycles to complete neurogenesis. It is also possible that the original pool of proliferative neurons is unchanged and that throughout neurogenesis, the proportion of daughter cells that elect to re-enter the cell cycle or terminally differentiate is somehow altered in these knockout mice. It is also possible that both circumstances are true.

The experimental protocol designed by Takahashi *et al*, 1994, 1996, is a valuable tool for assessing the kinetics of the proliferative population of the mouse PVE. It employs a distinct labeling protocol to measure the values for P, the proportion of proliferating cells that re-enter the cell cycle and Q, the proportion that terminally differentiate and become quiescent. P+Q=1 (see Figure 1. legend and *Materials and Methods* section for more detail). In this study we apply a modified version of this method to FGF2 -/- mice to determine P, Q, and P+Q. The P fraction is 1 and the Q is 0 at the outset of neurogenesis. As the neuronogenetic interval progresses the P approaches 0, while the Q increases to 1. The main objective of this present study is to test the effect of FGF2 gene deletion on the cytokinetic parameters, P and Q. Our hypothesis is that the absence of FGF2 causes premature exit of neuronal progenitors from the cell cycle resulting in an early progression of the Q fraction and early exhaustion of the progenitor population. This can result in a significantly decreased number of cortical neurons postnatally and in adulthood.

Materials and Methods

Animals

FGF2 knockout mice were obtained from the McLaughlin Research Institute (Zhou *et al*, 1998). Heterozygous (+/-) males were mated with either +/- or -/- females. The genotype was established using PCR as described (Zhou *et al*, 1998). The animals were maintained on a 12 hour (7:00 am to 7:00 pm) light dark schedule. Conception was ascertained by 9:00 am plug checks with the day of conception assigned E0.5.

Experimental Design/Analysis

This experimental design was modified from Takahashi *et al* 1994, 1996, (Figure 1). The purpose of this method was to determine in a two hour cohort of progenitor cells, the density of the proliferative and quiescent cells (∂p+q) and the density of quiescent cells only (∂q). Two S-phase markers ^{3}[H]thymidine (5mCi/gm body weight) and the thymidine analog 2-bromodeoxyuridine, (BrdU, 50mg/gm body weight) are used to distinguish these populations. At 8:00 am a single intraperitoneal injection of ^{3}[H]thymidine is administered to pregnant dams. The cells that entered the S-phase after the ^{3}[H]thymidine injection incorporate the ^{3}[H]thymidine into the DNA and become labeled. Two hours later, an intraperitoneal injection of BrdU is administered which is also incorporated into the DNA during the S-phase. This creates a two hour cohort of ^{3}[H]thymidine only labeled cells that exited the S-phase in the two hours before injection of the BrdU. Those ^{3}[H]thymidine labeled cells remaining in the S-phase during BrdU exposure become doubly labeled with ^{3}[H]thymidine and BrdU. The cells that entered the S-phase after BrdU injection become singly labeled with BrdU and those cells in the G_1 phase of the cell cycle are unlabelled.

Figure 1.
P & Q Protocol

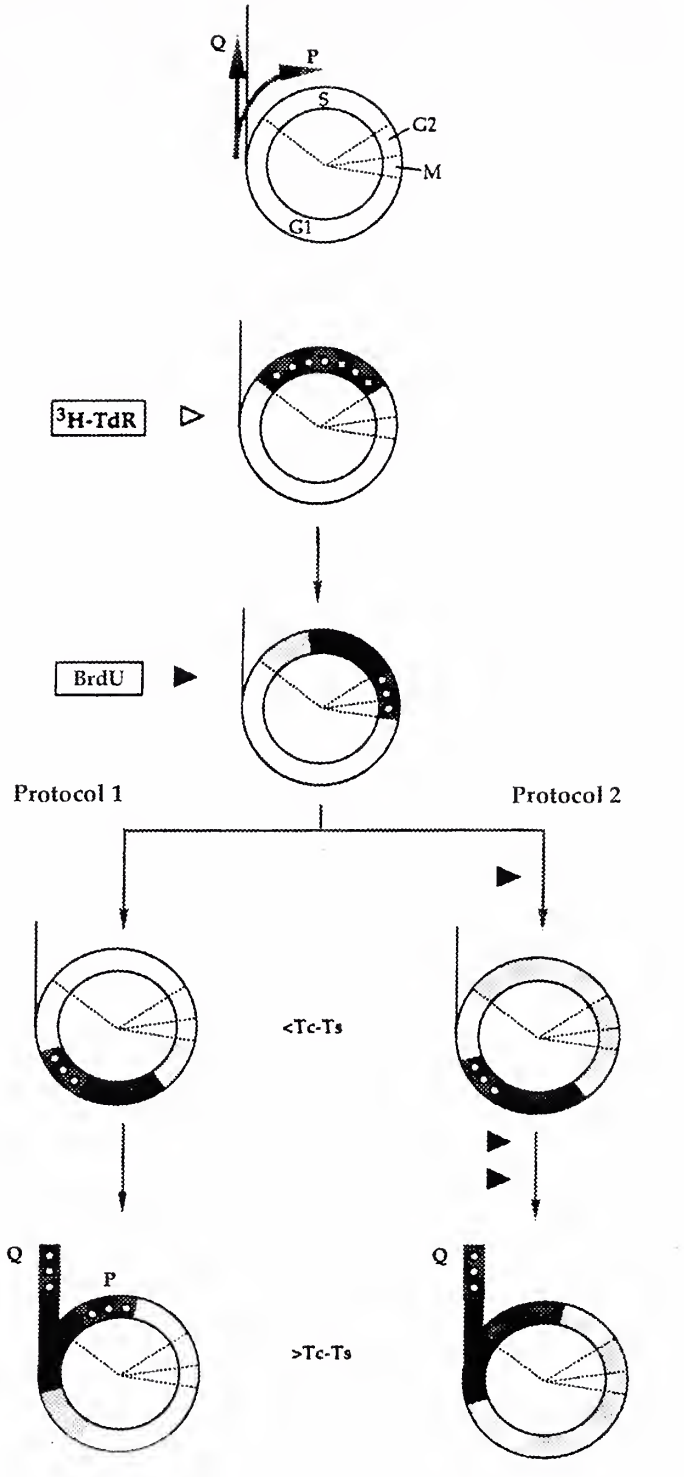


Figure 1. (Adapted from Takahashi *et al*, 1994) Cells in the S phase were labelled with a single injection of ^{3}[H]thymidine at 8:00 am (open arrowhead). At 10 am BrdU was injected creating a 2 hour cohort of cells labeled only with ^{3}[H]thymidine (shaded area with perforations). The cells remaining in S phase during BrdU injection are doubly labeled with ^{3}[H]thymidine and BrdU (darkly shaded area). The cells newly entering S phase are singly labeled with BrdU only (lightly shaded). **Protocol 1** is used to determine $\partial p+q$ or the density of proliferating and quiescent cells belonging to the 2 hour cohort. After the intial BrdU injection, no further injections are done and the animal is sacrificed at a time greater than the length of the cell cycle (Tc) minus the length of the S phase (Ts). At a time greater than Tc-Ts, the cells of the original 2 hour cohort will have either re-entered the S phase (p) or left the cell cycle to become quiescent (q). In both of these cases, the cells of interest are singly labeled with ^{3}[H]thymidine. Thus, the density of ^{3}[H]thymidine cells is equal to the sum of the density of cells in the proliferative and quiescent fractions of the 2 hour cohort ($\partial p+q$). **Protocol 2** is designed to determine the ∂q or density of quiescent cells. After the first BrdU injection there are a series of further injections 3 hours apart until 1/2 hour before the animal is sacrificed. This corresponds to a time greater than Tc-Ts and ensures that the cells of the original 2 hour cohort that have re-entered the S-phase become doubly labeled with BrdU and ^{3}[H]thymidine. The remaining ^{3}[H]thymidine only labeled cells comprise the ∂q . Thus, Protocol 1 and 2 yield $\partial p+q$ and $\partial_{q'}$ respectively. ∂p, and the P and Q fractions are mathematically derived from $\partial p+q$ and ∂q (see Table 1).

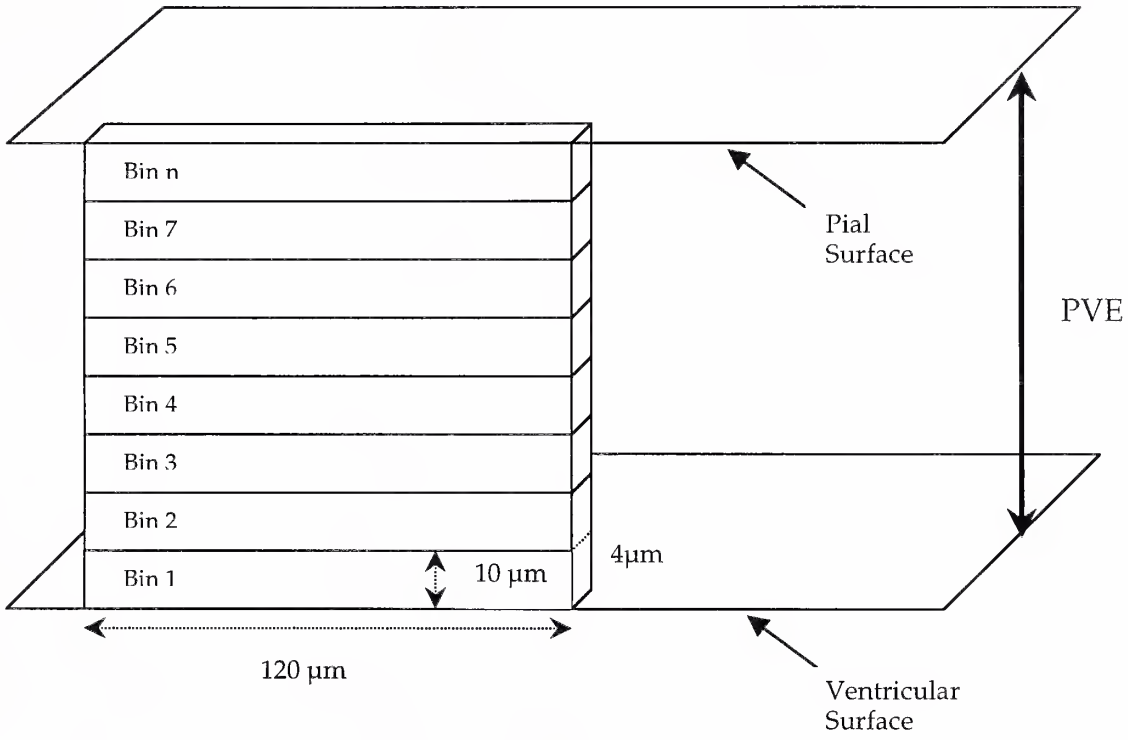
In Protocol 1, there are no further BrdU injections and the animal is sacrificed at a time greater than the length of the cell cycle minus the length of the S-phase (Tc-Ts). The net result is that the ^{3}[H]thymidine singly labeled cells from Protocol 1 represent the density of both the **p**roliferating and **q**uiescent cells of that original two hour cohort, or $\partial \mathbf{p+q}$.

In Protocol 2, after the initial BrdU injection which creates the two hour cohort of ^{3}[H]thymidine only labeled cells, there are subsequent injections of BrdU at three hour intervals until half hour before the animal is sacrificed which is equal to a time greater than Tc-Ts. Similar to Protocol 1 at the time of sacrifice, the cells of the original two hour cohort have either reentered the cell cycle or have exited. Those that reenter the cell cycle in Protocol 2 are exposed to the continued injections of BrdU and become doubly labeled with ^{3}[H]thymidine and BrdU. These doubly labeled cells are excluded from consideration. Only the singly labeled cells that exited the cell cycle are counted . These cells represent $\partial \mathbf{q}$, the density of quiescent cells of the original two hour cohort..

In both Protocol 1 and Protocol 2, the cells of interest are singly labeled with ^{3}[H]thymidine and are subsequently counted using light microscopy. The density of ^{3}Hthymidine labeled cells for Protocol 1 and Protocol 2 in control and FGF2 -/- animals was determined in sectors encompassing the entire dorso-ventral extension1 of the PVE. The sectors were divided into bins measuring 120μm in the anterioposterior dimension, 4μm in the mediolateral dimension, and 10μm in the inferiosuperior dimension. The bins are parallel to the ventricular surface with bin 1 starting at the ventricular border. In all cases the number of cells per bin are counted in ascending bins until the height of the PVE

is reached (see Figure 2). The number of cells per bin corresponds to a density measure since the bin is a unit of volume.

Figure 2. Bin Count Diagram



Measurements were conducted in both anterior and posterior regions of the same brain. Protocol 1 yielded $\partial \mathbf{p+q}$, or the density of proliferating and quiescent cells in all the bins for that area of the PVE. Protocol 2 yielded $\partial \mathbf{q}$ or the density of quiescent cells in the same area of a different animal. $\partial \mathbf{p}$, the density of proliferating cells was determined by subtracting $\partial \mathbf{q}$ from $\partial \mathbf{p+q}$ ($\partial p = \partial p + q - \partial q$).

Immunocytochemistry and Autoradiography

The embryos were removed by hysterotomy from dams anesthetized with a combination of ketamine (50 mg/kg) and xylazine (10 mg/kg). The embryos were measured from head to rump and fixed in 5% acetic acid in 70% ethanol for 2 hours then transferred to 70% ethanol overnight. The embryos were decapitated and dehydrated in graded ethanol solutions of 85%, 95%, and 100%. They were then cleared in toluene, embedded in paraffin and sectioned at 10 mm in the sagittal plane. The sections were stained for BrdU. After immunocytochemistry, the slides were dipped in a 50:50 mixture of Kodak NTB2 emulsion and 0.5% glycerol in a darkroom and stored at 4°C for 4 weeks. They were developed using Kodak D-19 developer for 2 minutes, transferred to distilled water for 15 seconds and then dipped in Kodak general purpose fixer for three minutes. The sections were counterstained with cresyl violet.

Results

Determination of ∂p+q, ∂q, and ∂p

In order to asses the effect of FGF2 gene deletion on the cytokinetic properties of neuronal precursor proliferation and quiescence in mouse PVE, we followed a modified protocol of Takahashi *et al* 1994, 1996, to obtain the P and Q fractions (see Figure 1. legend). P is equal to the proportion of neuronal precursor cells that continue to proliferate, while Q is the proportion of cells that exit the cell cycle to become quiescent throughout the neurogenetic interval.

The present experiment is conducted on brains of embryonic days 11.5, 13.5 and 15.5 (E11.5, E13.5, E15.5) which correspond to the beginning, middle and near end of the neurogenetic interval. The cytoarchitecture of the developing cerebral cortical wall changes throughout the neurogenetic interval. Prior to E14 the PVE, or pseudostratified ventricular epithelium predominates. This area contains dividing neuronal precursors. In the interval between E13 and E14, the PVE gives rise to a histologically and functionally separate region called the SPP, or secondary proliferative population. The SPP is the site of glial precursor cell proliferation. By E14 the PVE comprises 89% of the proliferative population and the SPP 11%, while at E16 the proportions are 65% and 35% respectively.

For this experiment, we are only considering the contribution of the PVE to the P and Q fraction. The first two time points, E11.5 and E13.5, contain no appreciable SPP. For the 15.5 time point, it was necessary to histologically distinguish the two regions and determine the P and Q fraction separately for the two proliferative populations.

The density of ^{3}Hthymidine only labeled cells was determined in a 120x4x10μm sector called a bin, using light microscopy (see Figure 2). Based

upon the experimental design outlined in Figure 1., two separate protocols were used to determine two distinct populations of ^{3}Hthymidine labeled cells in the PVE. In Protocol 1, ^{3}Hthymidine positive cells represent the density of P+Q cells (∂p+q), which is the density of proliferating and quiescent cells together. In Protocol 2 , ^{3}Hthymidine positive cells represent the density of Q cells (∂q) only. The density of proliferating cells (∂p) was obtained by subtracting ∂q from ∂p+q. The Proliferative and Quiescent fractions, P and Q respectively, were obtained by diving ∂q by ∂p+q and ∂p by ∂p+q. These formulas are given in Table 1.

Table 1. Determination of ∂p+q, ∂q, ∂p, P and Q

Protocol 1: **∂p+q**=density of proliferating and quiescent cells Protocol 2: **∂q**=density of quiescent cells (∂p+q and ∂q were experimentally determined)
∂p+q-∂q=∂p=density of proliferating cells **Q=∂q/∂p+q**=the proportion of cells exiting the cell cycle to become quiescent **P=∂p/∂p+q**=the proportion of proliferating cells **P+Q=1** (∂p, Q, and P were mathematically derived from ∂p+q and ∂q)

Counts were done for each of the three time points in both anterior and posterior regions of the same brain.

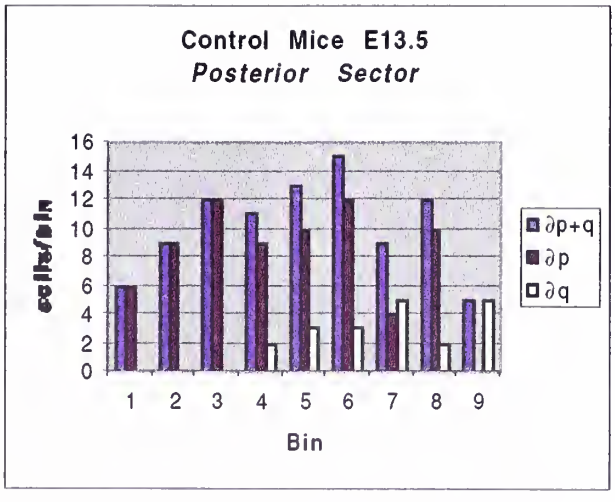
Distribution and Density of Proliferating and Quiescent Cells in Control and FGF2-/- Mice

Figure 3 shows the distribution of ∂p+q, ∂p and ∂q with respect to position in the developing cortical wall. Each sector was divided into bins and the density of ^{3}Hthymidine labeled cells within each 120x4x10μm bin is shown with respect to each other. The bins are parallel to the ventricular surface with bin 1 being adjacent to the ventricular border and bin 9 being near the pial surface (Figure 2). Bins 1-9, corresponding to the PVE, will be now considered the total volume of our sector for the purposes of this experiment. The density of cells of the labeled cohort within this volume (∂p+q) at E13.5 in the FGF2 -/- was 72 compared to 92 in the control which suggests that there is a lower density of cells in the FGF2 -/- mice than in control. There was a similar finding in the FGF2 -/- at E11.5 but not at E15.5.

Figure 3 shows the distribution of ∂p+q, ∂p, and ∂q in the posterior sector of E13.5 PVE. Although Fig. 3 shows that ∂q is more widely distributed throughout the PVE in FGF2 -/- mice, there was no overall consistent difference in the dorso-ventral distribution between control and FGF2 -/-, when all samples were considered, both anterior and posterior at the time points considered in this study.

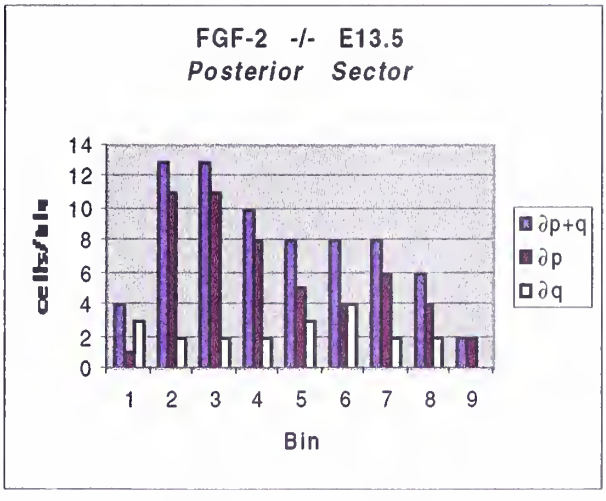
Figure 3. Distribution of ∂p+q, ∂p, and ∂q cells at E13.5.

A



∂p+q	∂p	∂q
92	71	21

B



∂p+q	∂p	∂q
72	52	20

Table 2. summarizes the P and Q data in both anterior and posterior regions of the same brain in FGF2 -/- and control animals.

Table 2. Cytokinetic Parameters in the PVE for E11.5-E15.5

∂p+q is the density of proliferative and quiescent cells in an area which spans the entire height of the PVE and is 120x4μm in length and depth. ∂p is the density of cells remaining in the cell cycle and ∂q is the density of cells exiting the cell cycle. P and Q are proportions of the aforementioned densities. P=∂p/∂p+q. Q=∂q/∂p+q.

A

FGF2-/- ANTERIOR	∂p+q	∂p	∂q	P	Q
E11.5	18	10	8	0.56	0.44
E13.5	109	79	30	0.72	0.28
E15.5	97	22	75	0.23	0.77
Control ANTERIOR					
E11.5	83	68	15	0.82	0.18
E13.5	129	91	38	0.71	0.29
E15.5	66	4	62	0.06	0.94

B

FGF2 -/- POSTERIOR	∂p+q	∂p	∂q	P	Q
E11.5	32	23	9	0.72	0.28
E13.5	72	52	20	0.72	0.28
E15.5	78	11	67	0.14	0.86
Control POSTERIOR					
E11.5	52	46	6	0.88	0.12
E13.5	92	71	21	0.77	0.23
E15.5	72	21	51	0.29	0.71

Progression of Q During Neurogenesis

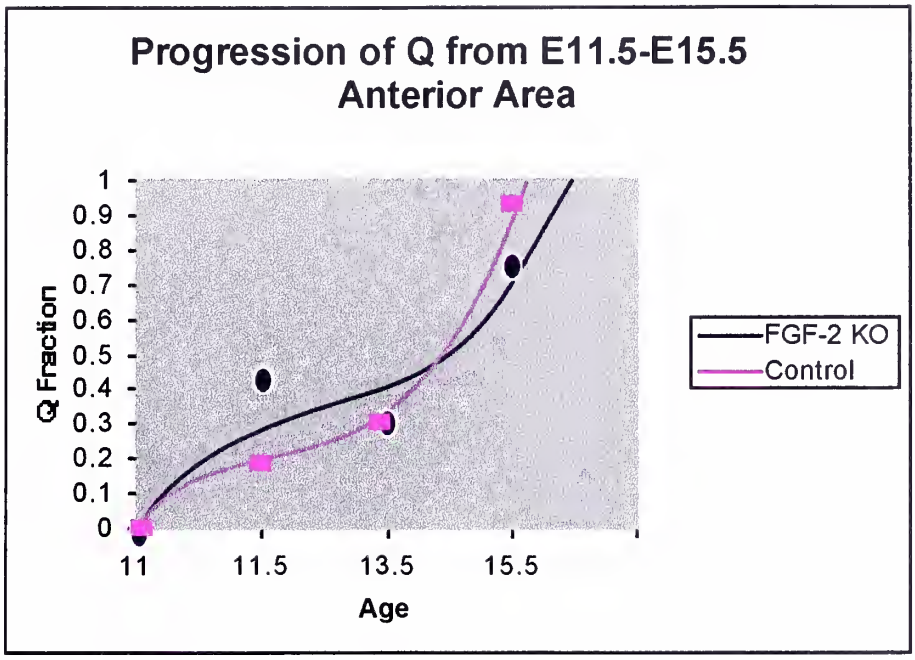
The Q fraction or proportion of cells which have exited the cell cycle proceeds from 0 at the beginning of neurogenesis and approaches 1 at the end of

neurogenesis (Takahashi *et al*, 1996). Figure 4 illustrates the progression of Q in anterior and posterior regions of the same brain for both FGF2 -/- and control

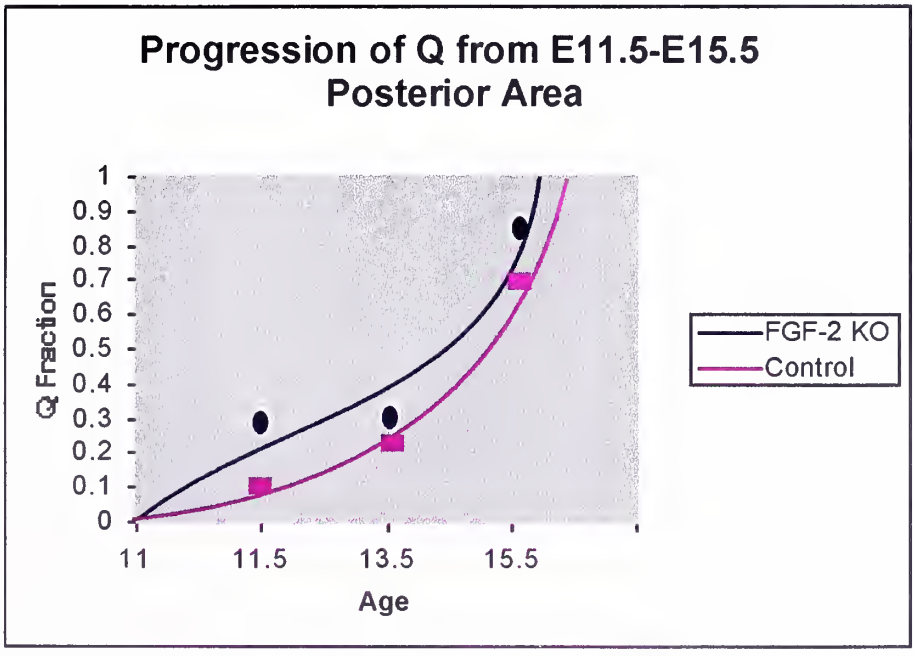
Figure 4. Progression of Q During Neurogenesis

Figure 4.A In the anterior region, the Q fraction is intially greater than P. After E13.5, the Q fraction falls below that of P. 4.B In the posterior region, the Q fraction is greater than P, at all measured time points during neurogenesis.

A



B

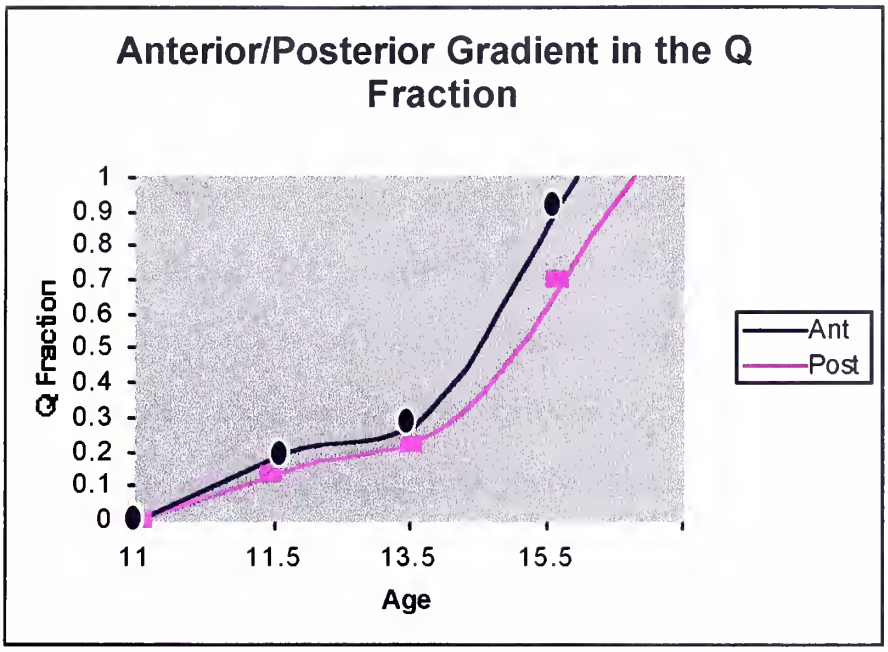


animals. The data for these graphs is shown in Table 1. Figure 4. A shows that in the anterior region of the E11.5, the Q fraction is higher in FGF2-/- than control, but after E13.5, it falls below that of the control value. This indicates that the proportion of cells exiting the cell cycle starts out greater than that for the control at the beginning of neurogenesis, but as neurogenesis proceeds, this proportion becomes smaller relative to the control. In Figure 4. A, the FGF2-/- Q fraction approaches 1 at a later time than the control. Similar to Figure 4. A, Figure 4.B begins with a relatively high value for Q, 0.28 versus 0.12 for control. This confirms that there is a loss of progenitor cells early in neurogenesis in FGF2-/- mice. However, the value for Q for the FGF2 -/- remains higher than that for the control throughout the neurogenetic interval in this graph. The time when Q approaches 1 is by definition the end of neurogenesis. This time is reached earlier for the FGF2-/- than for the control indicating that the absence of FGF2 induces a premature exhaustion of the proliferative population.

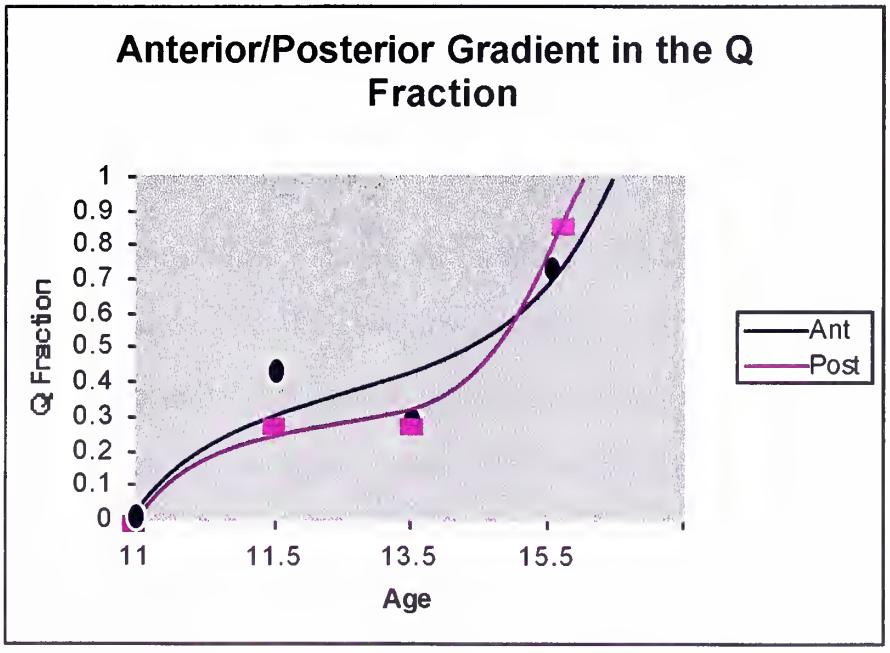
Figure 5. Progression of Q in Anterior Versus Posterior Regions

Figure 5.A At all time points during the neurogenetic interval, the proportion of cells exiting the cell cycle or Q, is greater in the anterior than in the posterior regions of the same brain in control mice. 5B. There is no consistent difference between the Q fraction of the anterior and posterior regions in FGF2 null mice.

A. Control



B. FGF2 -/-



Neurogenesis in the PVE is initiated anteriolaterally and proceeds posteriomedially (Bayer and Altman, 1991). This well described phenomenon is the result of a temporospatial gradient in neurogenesis. At all the time points during the neurogenetic interval, neurogenesis is more advanced in the anterior region compared to the posterior creating a gradient between anterior and posterior regions. If the gradient is intact, the measurement of post-mitotic cells or Q should be greater in the anterior portion of the PVE versus the posterior at the same point in time due to its relatively more advanced stage of development. Figure 5.A demonstrates that in control animals, the Q fraction is greater in the anterior region compared to the posterior. However there is no consistently elevated Q fraction in the anterior region throughout the neurogenetic interval in FGF2-/- animals (Figure 5.B), suggesting that the absence of the FGF2 has disrupted the temporospatial gradient in neurogenesis. The importance of the gradient is explained in the discussion.

Discussion

Previous studies performed by investigators in this lab have shown that FGF2 plays a significant role in the development of the cerebral cortex (Vaccarino *et al*, 1999). FGF2 null mice examined at adulthood revealed a nearly 50% decrease in cortical cell number, while E16 rats injected with FGF2 into the lateral cerebral ventricles showed an over 80% increase in cortical cell number at adulthood. In order to understand the mechanism of FGF2 in cortical development, three different approaches were undertaken. The first approach was directed at assessing what effect, if any, FGF2 had on progenitor cell survival. Studies performed in FGF2 microinjected rats showed no change in the amount of cortical

progenitor apoptosis. Similar studies will be conducted in FGF2 null mice.

The second approach involved determining the length of the cell cycle of FGF2 null mice versus control to determine if the absence of FGF2 had any effect on the length of the cell cycle. If the cell cycle length was increased then there would be fewer cycles within the neurogenetic interval to produce neurons. Cell cycle data revealed that there was no change in the length of the cell cycle between FGF2 null and control mice at E11.5.

The third approach, which was the main objective of this present study, was an attempt to test the effect of FGF2 gene deletion on the cytokinetic parameters, P and Q. Our hypothesis was that the absence of FGF2 causes premature exit of neuronal progenitors from the cell cycle resulting in an early progression of the Q fraction and early exhaustion of the progenitor population. This hypothesis predicts that Q should be higher in FGF2 -/- brains at all time points. At this point in our study, the interpretation of these data is complicated by the low number of animals which does not allow us to perform statistical analysis. However our preliminary data do provide some insights regarding the effect of this gene deletion on these cytokinetic parameters. Comparing anterior and posterior regions of the same brain had similar statistical limitations but was useful for the determination of the effect of FGF2 gene deletion on the temporospatial gradient of neurogenesis.

The Effect FGF2 Gene Deletion on the Progression of the Q Fraction.

In the anterior region (Figure 4.A), the Q fraction for the FGF2 -/- is elevated above that for the control at E11.5 but begins to wane after E13.5. The reason why the Q fraction is not consistently elevated in the FGF2 -/- mice is not

clear at present and more data are needed to elucidate the cause. It may suggest that the organism is attempting to compensate for the early decline in the P fraction toward the middle and end of neurogenesis. The effect of this compensation is graphically represented by a prolongation of the normal neurogenetic interval. If this compensation was inadequate, the result could be a decrease in the volume and total cortical cell number in the adult animal as demonstrated by previously obtained data.

While the anterior cortical region did not posses a consistent change in the Q fraction in FGF2-/- mice, in the posterior region we noticed an increase in Q throughout the neurogenetic interval. Figure 3.B illustrates a left shift in the Q curve for the FGF-/- at all time points. This indicates that throughout the neurogenetic interval, the absence of FGF2 has induced neuronal progenitors to prematurely exit the cell cycle. This also implies an early termination of neurogenesis which by extrapolation we determined to E17 in the control and E16 in the FGF2-/-. Shortening the neurogenetic interval is very likely to cause a severe decrease in the volume and cortical cell number in FGF2 -/- adult mice.

In Figure 4, both anterior and posterior cortical walls have strikingly high values for Q at E11.5. The increase in Q fraction at E11.5 implies a corresponding decrease in the P fraction. This is consistent with data recently obtained by our lab showing that at E11.5 in FGF2 -/- mice there is a decrease in the volume, total cell number, and number of proliferating cells of the PVE (Raballo, unpublished data). A lower proportion of proliferating cells in the PVE is expected to cause a decrease in the amplification of cortical progenitors due to a decrease in symmetric mitoses. Data obtained in this study also revealed a decrease in the density ($\partial p+q$) of the PVE in E11.5 and E13.5 FGF2 -/- samples (see Table 2.) All

these data taken together suggest that the effect of the gene deletion causes both a decrease in the proliferative pool before or at the very beginning of neurogenesis and induces early exhaustion of this already depleted pool.

PVE Gradient

It has long been known that neurogenesis proceeds in a non synchronous pattern in the neuroepithelium. It begins anteriolaterally and proceeds posteriomedially (Bayer and Altman, 1991) The gradient is dependent upon two factors, 1. A temporospatial delay in neurogenesis between anteriolateral and posteriomedial areas, and 2. A stepwise increase in G_1 with each successive integer cell cycle without a change in other cell cycle parameters. At a given time point during neurogenesis the length of the G1 phase of the cell cycle, T_{G1} in the anteriolateral region is greater than T_{G1} in the posteriomedial region of the same brain. Alterations in this gradient could lead to aberrant specification of cortical areas.

Data obtained in this study reveals that with respect to the Q fraction, which is an direct measurement of the kinetics of corticogenesis, there is an anterior posterior gradient in the control animals (Figure 5. A) Throughout the neurogenetic interval, the proportion of cells exiting the cell cycle is greater in the anterior versus posterior regions. Our data confirm that the progression of Q reflects the anterior to posterior and lateral to medial gradient of neurogenesis.

In the FGF2 -/- (Figure 5. B), Q is not consistently elevated in the anterior region throughout the neurogenetic interval. The Q values suggest that there is a disruption of the neurogenetic gradient in FGF2 -/- mice compared to the control mice. Previous data suggests that the absence of FGF2 has no effect on the length

of the cell cycle, therefore the disruption in the gradient would not be caused by an effect of FGF2 gene deletion on the length of the T_{G1}. The gene deletion may be disrupting the temporospatial delay causing neurogenesis to proceed more synchronously across the anterior/posterior dimensions of the PVE. If the gradient is responsible for creating a map of the cortex in the PVE, then altering the gradient could lead to aberrant cortical specification. Region specific markers for somatosensory, motor, or visual cortex could be used to further test this finding.

Conclusion

In this present study we have shown that the deletion of the gene for fibroblast growth factor 2 has adverse effects on the cytokinetic parameters P and Q in a murine model. This current data supports previous data which demonstrated that in FGF2-/- adult mice there was a 50% decrease in both the number of cortical neurons and cortical volume.

Our data also demonstrates that early in neurogenesis at E11.5 in FGF2-/- mice, the Q fraction is abnormally elevated in both posterior and anterior regions. This is consistent with previous data which showed that at E11.5 there was a decrease in the number of proliferating cells, and the volume of the PVE, which further suggests that the decrease in proliferating cells is due to their premature exit from the cell cycle.

We also found that in FGF2-/- mice at E11.5 and E13.5, there was a decrease in the density of the the cells of the proliferative cohort ($\partial p+q$) within thePVE.

These data taken together suggest that the effect of the gene deletion causes both a decrease in the proliferative pool of neuronal progenitors in the PVE and a premature depletion of this population.

In control animals, we were able to demonstrate the neurogenetic gradient with respect to the progression of the Q fraction. The gradient was disrupted in FGF2-/- animals suggesting that FGF2 is necessary for maintenance of the gradient. Disruptions in the gradient could lead to faulty area specification in the cortex leading to host of developmental problems.

The insights we have gained from this study have further elucidated the cytokinetics and molecular mechanisms of cortical development. This will allow us to better understand the pathophysiology of a host of genetic and congenital anomalies that may be associated with defects in FGF2 gene or gene product expression. These defects can have severe effects upon cognitive functioning leading to mental retardation and developmental delay. From this research and others similar endeavors we may be able to facilitate the development of novel treatment strategies.

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